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(54) Title: DNA SEQUENCE ENCODING A NOVEL MEMBER OF THE STEROID AND THYROID HORMONE RECEPTOR FAMILY

(57) Abstract

The invention provides a DNA sequence encoding a novel member of the steroid and thyroid hormone receptor family. The invention also relates to the isolation of said sequence and the expression of the encoded protein and chimeric proteins comprising all or part of the DNA or ligand-binding region of said hormone receptor. The invention furthermore provides antibodies directed to said proteins and diagnostic and therapeutic compositions for the diagnosis and therapy of steroid receptor-related diseases.

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DNA Sequence Encoding a Novel Member of the Steroid and Thyroid Hormone Receptor Family

The present invention relates to a DNA sequence encoding a novel steroid receptor. In particular, it relates to a novel DNA sequence encoding a novel steroid receptor involved in early T-cell activation, to expression plasmids containing said DNA, to host cells transformed by said expression plasmids, to methods for the production of said steroid receptor, to antibodies reacting with said protein, and to transgenic, non-human mammals containing said DNA sequence in their genome.
Furthermore, the present invention relates to diagnostic and pharmaceutical compositions containing said protein.

Steroid and thyroid hormones are known to coordinate complex molecular pathways involved in development, differentiation and physiological response to environmental stimuli (Evans, Science 240 (1988), pp. 889-895). Said hormones are thought to act through binding to specific intracellular receptor

receptor, said receptor exhibits increased affinity for certain DNA sequence elements (enhancers) associated with target genes. Interaction of the receptor-ligand complex with the DNA target sequence leads to altered gene expression.
The intracellular receptor molecules have been investigated in molecular detail in the past few years. The isolation and characterization of several steroid receptor cDNAs have led to the definition of a large family of putative regulatory proteins. This superfamily of steroid and thyroid receptor proteins includes the receptors for glucocorticoids, mineralocorticoids, estrogen, testosterone, progesterone, thyroid hormone, retinoic acid and vitamin D (Hazel et al., Proc. Natl. Acad. Sci. U.S.A. 85 (1988), pp. 8444-8448). Further members of said superfamily are various oncogenes such as v-erbA (O'Malley, Molecular Endocrinology 4 (1990), pp. 363-369).

Comparative analysis of the amino acid sequences of various receptor proteins revealed that the N-terminal region of steroid receptors is poorly conserved in both length and amino acid sequence. Said region is believed to be a transcriptional modulation domain. However, further downstream, three conserved domains have been identified.
Domain I is considered to comprise the DNA binding region. Said domain contains several conserved cysteins believed to form two zinc fingers.
Domains II and III are responsible for ligand binding and are located further towards the C-terminus of the molecule.

However, although steroid and thyroid receptor proteins display a commmon overall structure, said receptors mediate a great variety of different cellular responses.
Particularly important cellular events are those involved in human diseases. Widespread diseases such as cancer, autoimmune diseases or AIDS are all known to involve defects in

the normal signal transduction and/or transcription process. As explained above, steroid receptor proteins mediate between certain signals and transcription of their target genes. Thus, the receptor proteins might become the molecular key for developing successful and advantageous diagnostic and therapeutic applications.

Although several steroid receptor proteins exhibiting the above structural properties have been described, there is still an urgent need for the further isolation and characterization of novel steroid receptor proteins. It is known that many of the molecular causes for the most severe human diseases involve the immune system. Thus, it is particularly desired to search for steroid receptor proteins which display functionality in the immune system.

Consequently, the technical problem underlying the present invention is essentially to provide a DNA sequence encoding a novel member of the steroid and thyroid receptor superfamily involved in the modulation of the immune system.
The solution to the above technical problem is achieved by providing the embodiments characterized in the claims.

Other features and advantages of the invention will be apparent from the description of the preferred embodiments and the drawings. The sequence listing and drawings will now briefly be discussed.

SEQ ID NO. 1 shows the nucleotide sequence of the cDNA encoding the NOT (nuclear receptor of T-cells) steroid receptor protein (including 5' and 3' untranslated regions).

SEQ ID NO. 2 shows the derived amino acid sequence of the NOT steroid receptor protein.

Fig. 1: Detection of the NOT gene using the Southern Blot technique - Southern Blot of genomic DNA hybridized to the probe 2g25 A (nucleotides 1-1779).

lane 1: Hind III digest
lane 2: EcoRI digest
lane 3: BamHI digest
lane 4: NotI digest
lane 5: SacI digest
lane 6: Size marker; HindIII digested lambda DNA

Fig. 2: Detection of NOT mRNA in activated T-cells, activated cell lines, and unstimulated primary tissue by Northern Blot analysis.

Fig. 2A: Northern Blot of primary human T-cells hybridized to the probe 19g13 (nucleotides 1950-3427)

lane 1: unstimulated
lane 2: cycloheximide (CHX; 10 µg/ml), 3h
lane 3: A23187125 (125 ng/ml), CHX, 3h
lane 4: PMA (20 ng/ml), CHX, 3h
lane 5: PMA (20 ng/ml) + A23187 (125 ng/ml), CHX, 3h

Fig. 2B: Northern Blot of various cell lines activated by PMA (20 ng/ml) + A23187 (125 ng/ml), CHX, 3h and hybridized to the probe 19g13

lane 1: PEER (T-cell line)
lane 2: B95-8 (B-cell line)
lane 3: U937 (histiocytic lymphoma)
lane 4: Hs913T (fibroblast line)
lane 5: MRC-5 (fibroblast line)
lane 6: IMR-32 (neuroblastoma)
lane 7: HepG2 (hepatocell. carc.)
lane 8: HeLa (epitheloid carcinoma)

Fig. 2C: Northern Blot of unstimulated primary tissue hybridized to the probe 19g13

lane 1: lung
lane 2: spleen
lane 3: kidney
lane 4: brain
lane 5: thymus
lane 6: thyroid
lane 7: HeLa activated by PMA (20 ng/ml) + A23187 (125 ng/ml), CHX, 3h: positive control

Fig. 3: Western Blot of pSEM3A and PQE40A fusion proteins stained with anti-NOT serum (1:1000 dilution) - Verification of NOT polypeptide I (159-235)-specific antiserum by Western Blot analysis.

lane 1: Protein obtained from E.coli with pQE40 expression vector without NOT sequence (control)
lane 2: Fusion protein obtained with pQE40 expression vector with NOT peptide 159-235 fused to DHFR (pQE40A)
lane 3: Fusion protein obtained with pSEM3 expression vector with NOT peptide 159-235 fused to truncated ß-galactosidase (pSEM3A).

Fig. 4: Detection of NOT protein variants of 32 kDa, 62 kDa and 64 kDa with rabbit anti-NOT immune serum in a Western Blot of HeLa extracts - Western Blot of cytosolic and nuclear extracts from HeLa cells stained with anti-NOT serum generated against NOT polypeptide I (159-235)

lane 1: cytosolic extract of unstimulated HeLa cells
lane 2: cytosolic extract of HeLa cells stimulated with PMA (20 ng/ml) + A23187 (125 ng/ml), CHX (10 ug/ml), 3h
lane 3: Nuclear extract of HeLa cells stimulated with PMA (20 ng/ml) + A23187 (125 ng/ml), CHX, 3h

Fig. 5: Expression of NOT receptor protein in vivo. NOT protein was detectable in substantial amounts in sublining layer fibroblasts and in endothelial cells of synovial membranes of rheumatoid arthritis patients(dilution of NOT I serum 1:100).

The present invention relates to novel steroid receptor proteins and provides DNA sequences contained in the corresponding gene. Such sequences include in particular the sequences as illustrated in SEQ ID NO. 1, allelic derivatives of said DNA sequences and DNA sequences degenerated as a result of the genetic code for said sequence. They also include DNA sequences hybridizing under stringent conditions with the DNA sequence mentioned above and encoding at least the DNA binding domain or ligand binding domain of a steroid receptor which occurs in a substantial amount in T-cells activated by Ca^{++}-ionophore A23187 (125 ng/ml).

Although said allelic, degenerate and hybridizing sequences may have structural diversity due to naturally occurring mutations such as deletions, additions, inversions or substitutions, they will usually still exhibit essentially the same useful properties, allowing their use in basically the same diagnostic and therapeutic applications.

The DNA sequence of the invention can be obtained by the present invention. However, in case the obtained DNA sequence deviates in some positions from the claimed sequence, said particular DNA sequence can easily be generated by using site-directed mutagenesis on the obtained DNA sequence.

According to the present invention, the term "substantial amount" means that the amount of the corresponding mRNA is easily detectable by conventional Northern blot techniques as described in Sambrook et al., "Molecular Cloning: A

Laboratory Manual," 1989, Cold Spring Harbor Laboratory Press. The term "substantial amount" does not mean that the corresponding mRNA is only detectable by extremely sensitive means such as PCR.

According to the present invention, the term "hybridization" means conventional hybridization conditions. The term "hybridization" preferably refers to stringent hybridization (50% formamide, 4 x SSPE, 1% SDS, 0,5% BLOTTO and 5% PEG 35 000 at 42°C for 24 h) and washing conditions (final stringency washing of the blot in 0,1% SSC, 0,1% SDS at 50°C for 30 min.)

Preferred embodiments of the present invention are DNA sequences as defined above and obtainable from vertebrates, preferably from mammals such as pigs or cows, from rodents such as rats or mice, and in particular from primates such as humans.

A particularly preferred embodiment of the present invention is the DNA sequence as shown in SEQ ID NO. 1. To obtain said sequence, the inventors have constructed a cDNA library from human peripheral blood T-cells activated by PMA and ionophore. By differential screening, several novel T-cell activation genes were identified. Subsequent sequence analysis of one of the obtained clones revealed structural characteristics common to the steroid receptor family: a zinc-finger DNA binding domain and a ligand-binding domain containing a leucin-zipper motif. The protein encoded by said clone has been termed NOT (nuclear receptor of T-cells). The overall structure indicates that the NOT gene belongs to a new family of steroid receptor genes. The NOT receptor is encoded by an mRNA of 4.2 kb. Said mRNA codes for a protein

of 598 amino acids and is expressed in T-cells following stimulation by ionophore or PMA in the presence of cycloheximide.

The present invention also relates to DNA or RNA sequences capable of hybridizing to an RNA sequence derived from a DNA sequence of the present invention. In particular, the invention relates to an RNA or DNA sequence hybridizing to said 4.2 kb mRNA sequence.
Said sequences may prove particularly useful in experiments aiming to repress specific gene expression by introducing a DNA sequence coding for an antisense RNA or a ribozyme into the desired organism.

In the present invention, the cloning was achieved by differential screening of cDNA libraries. Once the DNA sequence has been cloned, the preparation of host cells capable of producing the steroid receptor protein and the production of said protein can easily be accomplished using known recombinant DNA techniques comprising constructing the expression plasmids encoding said protein and transforming a host cell with said expression plasmids, cultivating the transformant in a suitable culture medium and recovering the steroid receptor protein.
Thus, the invention also relates to recombinant molecules comprising DNA sequences as described above, optionally linked to an expression-control sequence. Such expression-control sequences may also include inducible expression control sequences. Such recombinant vectors may be particularly useful in the production of steroid receptor proteins in stably or transiently transformed cells. Several animal, insect, plant, fungal and bacterial systems may be employed for the transformation and subsequent cultivation process. Preferably, expression vectors which can be used in the invention contain sequences necessary for the replication in

the host cell and are autonomously replicable. It is also preferable to use vectors containing resistance genes which allow selection for transformed host cells. The necessary operations are well known to those skilled in the art.

It is another object of the invention to provide a host cell transformed by an expression plasmid of the invention and capable of producing a steroid receptor protein. Examples of suitable host cells include various eukaryotic and prokaryotic cells such as Bacillus or E. coli, plant cells such as tobacco, potato or Arabidopsis cells, animal cells such as insect cells or mammalian cells, preferably cells of the Mo-, COS- or CHO- cell line, and fungi such as yeast.

It is a further object of the invention to provide a process for the production of steroid receptor proteins. Such a process comprises cultivating said host cells being transformed by a DNA sequence of the present invention in a suitable culture medium and purifying the steroid receptor protein produced. Thus, this process will allow the production of a sufficient amount of the desired protein for use in medical treatments or diagnoses. Due to the nature of recombinant DNA technology, it will be understood that the protein as obtained by said process is free from polypeptides, proteins, or hormones with which it is naturally associated. Furthermore, depending on the host cell, the protein of the invention can be free from human, mammalian, bacterial, fungal, viral or plant proteins.

A further object of the present invention is to provide a steroid receptor protein encoded by the DNA sequences described above and displaying biological features such as ligand-activated transcription modulation, possibly relevant to therapeutic treatments. Putative ligands of the receptor may include classical steroid ligands or internal signal

transduction molecules. The above-mentioned feature of the protein might vary depending on the formation of homomeres or heteromeres. Such structures may prove useful in clinical applications as well.

A particularly preferred embodiment of the present invention is a receptor protein comprising the amino acid sequence as depicted in SEQ ID NO. 2 or a part thereof.

Further preferred embodiments are polypeptides derived from the steroid receptor protein of the invention. Particularly preferred are polypeptides which comprise a peptide fragment having one of the amino acid sequences selected from the group consisting of:

(a) SerSerProGlnGlyAlaSerProAlaSerGlnSerTyrSerTyrHisSer SerGlyGluTyrSer

(b) ValLysPheSerMetAspLeuThrAsnThrGluIleThrAlaThrThrSer

(c) AsnTyrSerThrGlyTyrAspValLysProProCysLeuTyrGlnMetPro LeuSerGlyGlnGlnSerSerIleLysValGluAspIleGlnMetHisAsn TyrGlnGlnHisSerHisLeuProProGlnSerGluGluMetMetProHis SerGlySerValTyrTyrLysProSerSerProProThrProThrThrPro GlyPheGlnValGlnHisSerProMetTrpAspAspProGlySerLeuHis AsnPheHisGlnAsnTyrValAlaThrThrHisMetIleGluGlnArgLys ThrProValSerArgLeuSerLeuPheSerPheLysGlnSerProProGly ThrProValSerSerCysGlnMetArgPheAspGlyProLeuHisValPro MetAsnProGluProAlaGlySerHisHisValValAspGlyGlnThrPhe AlaValProAsnProIleArgLysProAlaSerMetGlyPheProGlyLeu GlnIleGlyHisAlaSerGlnLeuLeuAspThrGlnValProSerProPro SerArgGlySerProSerAsn

(d) GlnGluProSerProProSerProProValSerLeuIleSerAlaLeuVal ArgAlaHisValAspSerAsnProAlaMetThrSerLeuAspTyrSerArg PheGlnAlaAsnProAspTyrGlnMetSerGlyAspAspThrGlnHisIle

(e) ThrGlySerMetGluIleIleArgGly

(f) ValGluPheSerSerAsnLeuGlnAsnMetAsnIleAspIleSerAlaPhe SerCysIleAlaAlaLeuAlaMetValThrGlu

(g) LysIleValAsnCysLeuLysAspHisValThrPheAsnAsnGlyGlyLeu AsnArgProAsnTyrLeuSerLys

The present invention also relates to chimeric proteins containing, in their amino acid sequences, all or part of an amino acid sequence encoded by the DNA sequences of the present invention. In particular, the invention relates to a chimeric protein comprising the ligand-binding region of the steroid receptor or a part thereof, optionally in combination with all or part of the DNA binding region. Preferably, the chimeric proteins contain all or part of the ligand-binding region of the steroid receptor protein with mutations introduced in the ligand-binding region aimed to change the ligand specifity of the steroid receptor protein.

The invention also relates to a chimeric protein comprising the ligand-binding region of the steroid receptor or a part thereof and a DNA binding region of a different steroid receptor. The chimeric proteins of the invention may be particularly useful in the search for physiological ligands, ligand analogues or blocker substances of the NOT receptor. In particular, said proteins may prove useful in drug-screening experiments and in diagnostic and clinical applications.

It is another object of the present invention to provide antibodies which specifically react with a steroid receptor protein or a part thereof encoded by the DNA sequences of the present invention. A preferred embodiment of the present invention relates to monoclonal antibodies directed specifically to a steroid receptor protein or a part thereof encoded by the DNA sequences of the present invention.

Yet another object of the present invention is to provide a particularly sensitive process for the detection of aberrant

variations of steroid receptors. Said process uses specifically selected primers to amplify coding regions of the steroid receptor gene.

It is another object of the present invention to provide pharmaceutical and diagnostic compositions containing a therapeutically or diagnostically effective amount of the steroid receptor protein, a DNA or RNA sequence encoding said protein, or an antibody directed to said protein. Optionally, such a composition comprises a pharmaceutically acceptable carrier. Such a therapeutic composition can be used in treating diseases such as cancer, AIDS, and various immunodeficiency-related diseases. Moreover, the diagnostic or pharmaceutical composition may include modified steroid receptor proteins or parts thereof in which functionally important amino acid alterations have been effected in order to modify DNA-binding activity or ligand-binding activity. Such alterations may include amino acid substitutions, deletions, additions, or inversions.

The diagnostic composition may prove particularly useful in determining the presence and/or quantity of steroid receptors in tissues or body fluids such as blood or lymphe.

The pharmaceutical composition comprising the proteins of the invention can also be used prophylactically. Furthermore, the application of the composition is not limited to humans but can also include animals, in particular domestic animals.

A further object of the present invention is to provide non-human mammalians which contain a DNA sequence according to the present invention in their genomes, with the proviso that said DNA sequence is a DNA sequence which does not naturally occur in said host organism. Furthermore, the invention

relates to non-human mammalians in which the naturally occurring steroid receptor genes encoding the protein of the invention have been made non-functional or have been altered in their ligand or DNA binding specificity. In particular, the availability of known transformation systems and the DNA sequence of the invention allows the construction of recombinant organisms where the endogenous steroid receptor gene is replaced by a mutated, for instance a functionless, copy. Most preferably, this object can be achieved by transformation-mediated gene disruption which involves homologous recombination between the endogenous gene and the transformed mutated gene copy. Such obtained non-human mammalians are particularly useful in studying the developmental and physiological role of the steroid receptor of the present invention. Most particularly, said studies may prove useful in developing advantageous and novel approaches for medical applications.

The following examples illustrate the invention but should not be construed as limiting the invention.

Example 1: Cloning of the steroid receptor NOT (Nuclear receptor Of T-cells)

Generation of a cDNA library from activated T-cells

Peripheral blood mononuclear human cells were isolated from buffy coats obtained from healthy donors by Ficoll-Hypaque gradient centrifugation. Passage of the mononuclear cells over a nylon wool column (Julius et al., Eur.J.Immunol. 3 (1973), pp. 645-649) resulted in a 95 % pure peripheral blood (PB) T-cell population. The PB T-cells were stimulated by PMA (20 ng/ml) + A23187 (125 ng/ml) for 2 h in the presence of cycloheximide (CHX, 10 ug/ml) in complete medium (see below). From the activated T-cells total cellular RNA was isolated

(Chirgwin et al., Biochemistry 18 (1979), pp. 5294-5299) and enriched for poly(A)-RNA by two passages over an oligo(dT)-column (Aviv et al. Proc.Natl.Acad.Sci.U.S.A. 69 (1972), pp. 1408-1412). Using oligo$(dT)_{15-17}$ primers and AMV reverse transcriptase, the poly(A)-RNA was transcribed into single stranded cDNA (Sambrook et al., "Molecular Cloning: A Laboratory Manual," 1989, Cold Spring Harbor Laboratory Press). Double stranded cDNA was obtained according to the method of Gubler and Hoffman (Gene 25 (983),pp. 263-269), blunt-ended with the T4-polymerase, and methylated with EcoRI-methylase. After ligation of EcoRI-linkers, the cDNA was treated with EcoRI and size fractionated by gel electrophoresis. cDNA fragments > 500 bp were cloned into the lambda gt10 phage vector (Stratagene).

Differential screening for activation genes, establishment of a gene collection at the cDNA level

The generated cDNA library was differentially screened for activation genes, i.e. genes strongly upregulated after cell activation (1-2% of all transcribed genes in stimulated cells). For screening, purified PB T-cells were either left unstimulated or activated by PMA (20 ng/ml) + A23187 (125 ng/ml) for 2 h in the presence of CHX. From both populations poly(A) RNA was isolated and transcribed into ^{32}P-labeled cDNA using oligo(dT)-primers, AMV reverse transcriptase and ^{32}P-dCTP according to standard methods (Sambrook et al., "Molecular Cloning: A Laboratory Manual," 1989, Cold Spring Harbor Laboratory Press). Replica plaque lifts of 120 000 clones from the lambda gt10 library were then differentially screened with the ^{32}P-labeled cDNA from the activated T-cells and in parallel with ^{32}P-labeled cDNA from resting T-cells (hybridization conditions: 50% formamide, 5X SSPE, 0.1% SDS, 5% PEG, probe at $1-2x10^6$ cpm/ml). 1 000 lambda gt10 clones hybridizing only with the cDNA from activated T-cells were

thus identified ("pre-collection"). Inserts from single lambda gt10 clones of the pre-collection were subsequently subcloned into the Bluescript II SK plasmid vector (Stratagene) and cross-hybridized against all remaining clones of the pre-collection. Thus the redundancy in the pre-collection was eliminated and a final collection of 100 distinct cDNA clones established ("activation gene collection").

Identification of the NOT receptor using a multigene analysis system

The 100 distinct cDNAs of the activation gene collection were dot-blotted as denatured plasmid DNA onto a nylon membrane. The membrane was hybridized with ^{32}P-labeled single stranded cDNA obtained from reverse transcribed RNA originating from T-cells activated by PMA (20 ng/ml) + A23187 (125 ng/ml) either for 2 h or for 24 h. Only 5 clones of the activation gene collection gave a signal in both hybridizations, indicating an ongoing transcription of the corresponding gene both at 1 h and 24 h after T-cell activation. All 5 cDNAs were partially sequenced by the dideoxy chain termination method (Sanger et al. Proc.Natl.Acad.Sci.U.S.A. 74 (1977), pp. 5463-5467). One of the cDNAs (full lenght clone 2g25) was found to contain structural features common to steroid receptors (steroid zinc-finger, ligand-binding domain). The sequence of the full length clone 2g25 encoding the NOT receptor is shown in SEQ ID No. 1. Clone 2g25 was used to isolate from the original cDNA library a partial length clone 19g13 (nucleotides 1950-3427).

Example 2: Detection of the steroid receptor at the genomic and mRNA levels

Southern analysis

Five ug of genomic placental DNA were digested with appropriate restriction enzymes, size separated on a 0.7% agarose gel, and transferred onto a nylon membrane. The 2g25A probe (nucleotides 1-1779) was nick-translated to a specific activity of 2 x 10^8 cpm/ug DNA by standard methods (Rigby et al. J.Mol.Biol. 113 (1977), pp. 237-251). The membrane was hybridized with the probe (1-2x10^6 cpm/ml) in 50% formamide, 4 x SSPE, 1% SDS, 0,5% BLOTTO and 5% PEG 35 000 at 42°C for 24 h. Blots were washed including a final stringency step (0,1% SSC, 0,1% SDS at 50°C for 30 min.) (see Fig. 1).

Northern analysis

Human T-cells and various cell lines were cultured in "complete medium" (RPMI 1640 medium supplemented with 10% heat inactivated FCS, 25 mM Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin, 50 uM 2-mercaptoethanol at 37°C in a humidified atmosphere of 5% CO_2 in air). Cells were stimulated with PMA (20 ng/ml) + A23187 (125 ng/ml) in the presence or absence of cycloheximide (10 ug/ml). Five ug of total cellular RNA were size fractionated on a 1.1% formaldehyde agarose gel and vacuum blotted onto a nylon membrane (Kroczek et al. Anal.Biochem. 184 (1990), pp. 90-95). Labeling of the 19g13 probe (nucleotides 1950-3247) and hybridization was as described under Southern analysis. The expression of NOT receptor mRNA in T-cells, various cell lines, and primary tissue is shown in Fig. 2. The mRNA length was typically 4.2 kb, in primary osteoblasts and brain tissue the 19g13 probe hybridized primarily to an mRNA of 2.7 kb.

In situ hybridization

NOT 2g25A cDNA (nucleotides 1-1779) cloned into bluescript SK+ vector (Stratagene), was used to generate sense and antisense ^{35}S-UTP riboprobes with a specific activity of $3x10^8$/ug, which were subsequently reduced in size by alkaline hydrolysis (Cox et al., Dev.Biol. 101 (1984), pp. 485-502). The probes were applied at a concentration of $6x10^7$ cpm/ml hybridization mixture. Cryostat sections were mounted on aminopropylsilan-coated slides, fixed in 4% para-formaldehyde in PBS and dehydrated. The dry sections were acetylated in 0.1 M triethanolamine, 0.25% acetic anhydride for 10 min, washed in 0.2 X SSC, and preincubated for 2 h at 45°C in 50% formamide, 0.6 M NaCl, 2.5 X Denhardt's solution, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS, and 0.15 mg/ml t-RNA (solution I). The sections were then incubated for 16 h at 45°C with the riboprobes in solution I, after addition of dextran sulfate to 10% final. The slides were washed in 50% formamide, 1X SSC, and 1mM DTT at 50°C, then treated with RNAse A (10 ug/ml), washed 4x in 0.1X SSC at 60°C, dehydrated, and finally coated with Kodak NTB-2 emulsion. Exposure time was 10-14 days. The specificity of the signals was verified by using sense riboprobes in parallel. The large majority of signals was found in fibroblasts and endothelial cells in synovial membranes and in a subcompartment of endothelial cells in spleen.

Example 3: Isolation of the NOT gene

A genomic library from human placenta (Lambda Fix II, Stratagene) was screened with the 2g25A probe at stringent hybridization conditions (formamide 50%, SSPE 5x, SDS 0.1%, PEG 35000 5%) at 52°C. A genomic clone of 11 kb containing the NOT gene was isolated and verified by restriction mapping.

Example 4: Expression of NOT receptor protein fragments in E. coli and generation of antibodies against the NOT receptor

NOT cDNA was digested with the restriction enzymes MscI (at position 790) and PstI (at position 1 025) and the resulting cDNA fragment coding for the polypeptide I (position 159-235)

ThrThrHisMetIle
GluGlnArgLysThrProValSerArgLeuSerLeuPheSerPheLysGlnSerProPro
GlyThrProValSerSerCysGlnMetArgPheAspGlyProLeuHisValProMetAsn
ProGluProAlaGlySerHisHisValValAspGlyGlnThrPheAlaValProAsnPro
IleArgLysProAlaSerMetGlyPheProGlyLeu

was inserted into the prokaryotic expression vector pSEM3 bearing a truncated ß-galactosidase sequence (Knapp et al., Biotechniques 8 (1990), pp. 280-281) via the SmaI and PstI cloning sites (pSEM3A). The cDNA fragment was also subcloned from the pSEM3A construct into the dihydrofolate-reductase fusion protein expression vector pQE40 (Qiagen), using the KpnI and HindIII restriction sites resulting in vector pQE40A.

A second NOT fragment was obtained by digestion of the cDNA with the restriction enzymes ScaI (at position 1 267) and SalI (at position 1 435) and the resulting cDNA fragment coding for the polypeptide II (position 318-372):

CysArgPheGlnLysCysLeuAlaValGlyMetValLysGluValValArgThrAspSer
LeuLysGlyArgArgGlyArgLeuProSerLysProLysSerProGlnGluProSerPro
ProSerProProValSerLeuIleSerAlaLeuValArgAlaHis

was inserted into the pSEM3 vector via the SmaI and SalI cloning sites resulting in vector pSEM3B. The frament was also subcloned from the pSEM3B into pQE40 using the KpnI and HindIII restriction sites (pQE40B).

E. coli BMH 7118 (Messing et al., Proc.Natl.Acad.Sci.U.S.A. 74 (1977), pp. 3642-3646) were made competent by the Hanahan method (J.Mol.Biol. 166 (1983), pp. 557-580) and transformed with the pSEM3A or pSEM3B constructs. BMH 7118 cells containing the constructs were grown to an O.D. of 0.6 and ß-galactosidase fusion proteins were induced by 1 mM IPTG. Further purification of inclusion bodies was according to MARSTON (Biochem.J. 240 (1986), pp. 1-12). The inclusion bodies were dissolved in 8 M urea and then desalted on a PD-10 column (Pharmacia). Approximately 500 ug of respective fusion proteins were mixed at a 1:1 (v/v) ratio with complete Freund's adjuvant and injected into rabbits s.c. at multiple sites. After several boosts with incomplete Freund's adjuvant the rabbits were bled and NOT polypeptide I or II specific antisera obtained. The specificity of these antisera was tested by Western Blot analysis against pQE40A and pQE40B fusion proteins which were obtained from IPTG-induced E.coli. (see Fig. 3).

Example 5: Detection of the NOT receptor at the protein level in primary T-cells and cell lines.

Cytosol of HeLa cells or T-cells was prepared by disrupting the cells in a glass-teflon motor-driven homogenizer (120 stokes) in TEM-medium (10 mM Tris, 1 mM EDTA, pH 7.4 containing 20 mM sodium molybdate) plus a cocktail of protease inhibitors (1 ug/ml pepstatin A, 2 ug/ml leupeptin, 2 ug/ml aprotinin, and 100 ug/ml bacitracin; Wei et al. Biochemistry 26 (1987), pp. 6262-6272). After low speed (15 min, 1,500 g) and high speed (30 min, 100,000 g) centrifugation the cytosol was concentrated by precipitation in 50% ammonium sulfate followed by desalting in a NAP-5 column (Pharmacia).

For nuclear extracts, cells were disrupted as described above, except that the buffer was TEG (10 mM Tris, 1 mM EDTA, pH 7.4 plus 10% glycerol). After low speed centrifugation, the pellet was extracted with 0.4 M KCl, and the suspension was then centrifuged at 100,000 g for 60 min to obtain the nuclear extract.

Ten ug of total protein were size separated on a 10% polyacrylamide gel according to Laemmli (Nature 227 (1970), pp. 680-685). The protein was transferred electrophoretically from the polyacrylamide gel onto a nitrocellulose membrane using a standard semidry blotting system (Renner) for 1 h at 0.8 mA/cm^2. After blocking of nonspecific binding with 5 % (w/v) nonfat dry milk and washing with PBS, the membrane was incubated overnight with 1:40 dilution of rabbit anti-NOT immune serum. After an additional wash step with PBS the blot was incubated with goat anti-rabbit immunglobulin coupled to peroxidase (1:8 000 dilution). The signal was developed using 4-chloro-1-naphthol as substrate according to standard methods (see Fig. 4).

Example 6: Detection of NOT receptor protein in vivo

Cryostat sections of inflamed synovial tissue from rheumatoid arthritis patients were fixed for 10 min in acetone. Immunohistology was performed on the sections using the alkaline phosphatase antialkaline phosphatase (APAAP) technique (Cordell et al., J.Histochem.Cytochem. 32 (1984), pp. 219-229. The section were incubated with anti-NOT serum at a dilution of 1:100 in Tris-buffered saline. After a washing step a $F(ab)_2$ goat anti-mouse antibody was applied for 1 h (1:20 in TBS/1% BSA). APAAP complex at a dilution of 1:100 (in TBS/1% BSA) was added for 30 min after repeated washing steps. The alkaline phosphatase was developed in a solution

containing 3 mg naphthol-AS-MX-phosphate in 200 ul N,N-dimethylformamide, 10 mg Fast Red TR, and 2,4 mg levamisole in 10 ml TBS, pH 8.6 for 30 min. The sections were counterstained with Mayer's hematoxylin (see Fig. 5).

SEQ ID NO: 1

SEQUENCE TYPE: Nucleotide

SEQUENCE LENGTH: 3427 base pairs

STRANDEDNESS: Single

TOPOLOGY: Linear

MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE:

ORGANISM: Human

IMMEDIATE EXPERIMENTAL SOURCE: T-Cells

PROPERTIES: Steroid receptor protein

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GCCCGGGCTC CCAGAGGGAA CTGCACTTCG GCAGAGTTGA ATGAATGAAG AGAGACGCGG    180
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SEQ ID NO: 2

SEQUENCE TYPE: Amino acid

SEQUENCE LENGTH: 598 residues

STRANDEDNESS:

TOPOLOGY: Linear

MOLECULAR TYPE: Peptide

ORIGINAL SOURCE:

ORGANISM:

IMMEDIATE EXPERIMENTAL SOURCE:

PROPERTIES: Steroid receptor protein

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MetProCysValGlnAlaGlnTyrGlySerSerProGlnGlyAlaSerProAlaSerGln    20
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SerThrPheMetAspAsnTyrSerThrGlyTyrAspValLysProProCysLeuTyrGln    80
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LeuValProProProAlaIleIleAspLysLeuPheLeuAspThrLeuProPhe          598
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Claims

1. A DNA sequence encoding a steroid receptor selected from the group consisting of:
   (a) the DNA sequence of SEQ ID NO. 1 or a part thereof;
   (b) a DNA sequence hybridizing to the DNA sequence of (a) and encoding at least the DNA binding domain or ligand-binding domain of a steroid receptor which occurs in a substantial amount in primary T-cells activated by the Ca^{2+}-ionophore A23187;
   (c) a DNA sequence which is degenerate with respect to a DNA sequence according to (a) or (b).

2. A DNA or RNA sequence hybridizing to an RNA sequence derived from a sequence according to claim 1.

3. A recombinant DNA molecule containing a DNA sequence according to claim 1.

4. The recombinant DNA molecule according to claim 3 wherein said DNA sequence is under the control of a promoter allowing its expression in a desired host cell.

5. A host cell containing a recombinant DNA molecule according to claim 4.

6. The host cell according to claim 5 which is a bacterial cell, a yeast cell, an insect cell, a plant cell or a mammalian cell.

7. A method for the production of a steroid receptor protein comprising the cultivation of a host cell according to claim 5 or 6 under conditions appropriate for expression of said DNA sequence and recovering said protein from the culture.

8. A steroid receptor protein encoded by a DNA sequence according to claim 1.

9. The steroid receptor protein according to claim 8 having the amino acid sequence of SEQ ID NO. 2.

10. A polypeptide derived from a steroid receptor protein according to claim 8 or 9 wherein the polypeptide comprises a peptide fragment having one of the amino acid sequences selected from the group consisting of:

(a) SerSerProGlnGlyAlaSerProAlaSerGlnSerTyrSerTyrHisSer SerGlyGluTyrSer

(b) ValLysPheSerMetAspLeuThrAsnThrGluIleThrAlaThrThrSer

(c) AsnTyrSerThrGlyTyrAspValLysProProCysLeuTyrGlnMetPro LeuSerGlyGlnGlnSerSerIleLysValGluAspIleGlnMetHisAsn TyrGlnGlnHisSerHisLeuProProGlnSerGluGluMetMetProHis SerGlySerValTyrTyrLysProSerSerProProThrProThrThrPro GlyPheGlnValGlnHisSerProMetTrpAspAspProGlySerLeuHis AsnPheHisGlnAsnTyrValAlaThrThrHisMetIleGluGlnArgLys ThrProValSerArgLeuSerLeuPheSerPheLysGlnSerProProGly ThrProValSerSerCysGlnMetArgPheAspGlyProLeuHisValPro MetAsnProGluProAlaGlySerHisHisValValAspGlyGlnThrPhe AlaValProAsnProIleArgLysProAlaSerMetGlyPheProGlyLeu GlnIleGlyHisAlaSerGlnLeuLeuAspThrGlnValProSerProPro SerArgGlySerProSerAsn

(d) GlnGluProSerProProSerProProValSerLeuIleSerAlaLeuVal ArgAlaHisValAspSerAsnProAlaMetThrSerLeuAspTyrSerArg PheGlnAlaAsnProAspTyrGlnMetSerGlyAspAspThrGlnHisIle

(e) ThrGlySerMetGluIleIleArgGly

(f) ValGluPheSerSerAsnLeuGlnAsnMetAsnIleAspIleSerAlaPhe SerCysIleAlaAlaLeuAlaMetValThrGlu

(g) LysIleValAsnCysLeuLysAspHisValThrPheAsnAsnGlyGlyLeu AsnArgProAsnTyrLeuSerLys

11. A chimeric protein comprising the protein or polypeptide sequence according to any one of claims 8 to 10 or a part thereof.

12. The chimeric protein according to claim 11 comprising the ligand binding region of the steroid receptor or a part thereof.

13. The chimeric protein according to claim 12 which comprises the ligand-binding region of the steroid receptor or a part thereof and a DNA binding region of a different steroid receptor.

14. The chimeric protein according to claim 12 or 13 which comprises mutations changing the ligand specifity of said steroid receptor.

15. An antibody specifically reacting with the steroid receptor protein according to claim 8 or 9 or with a polypeptide according to claim 10.

16. The antibody according to claim 15 which is a monoclonal antibody.

17. A diagnostic composition for the diagnosis of steroid receptor related diseases containing a DNA sequence according to claim 1, an RNA hybridizing to said DNA sequence, a protein or polypeptide according to any one of claims 8 to 14 or an antibody according to claim 15 or 16.

18. A diagnostic composition according to claim 17 for the determination of steroid receptors in tissues and body fluids.

19. A pharmaceutical composition for the treatment of steroid receptor-related diseases containing a DNA sequence according to claim 1, an RNA sequence hybridizing to said DNA sequence, a protein or polypeptide according to any one of claims 8 to 14 or an antibody according to claim 15 or 16, optionally together with a pharmaceutically acceptable carrier.

20. Use of a DNA sequence according to claim 1 or a part thereof as a primer to specifically amplify coding regions of a steroid receptor gene.

21. A transgenic, non-human mammalian containing in its genome a DNA sequence according to claim 1 or 2, wherein said DNA sequence is a DNA sequence which does not naturally occur in said host organism.

22. A non-human mammalian characterized in that the genes of said animal encoding a steroid receptor protein according to claim 7 or 8 have been made non-functional by transformation-mediated gene disruption.

Fig. 1

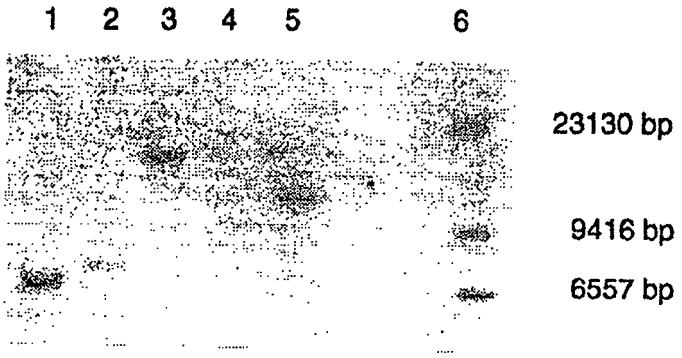

Fig. 2

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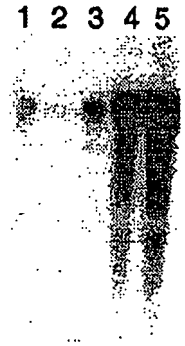

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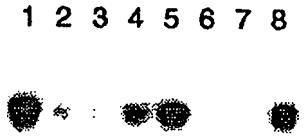

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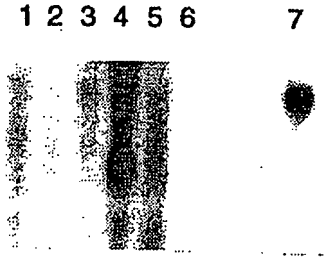

Fig. 3

Fig. 4

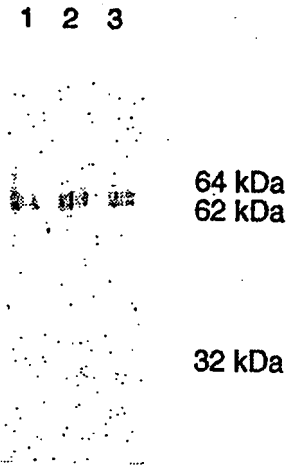

Fig. 5